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RESEARCH ARTICLE

Hake fish bone as a calcium source for efficient bone mineralization

Lisa Flammini^{a*}, Francesca Martuzzi^{b*}, Valentina Vivo^a, Alessia Ghirri^b, Enrico Salomi^a, Enrico Bignetti^b and Elisabetta Barocelli^a

^aDepartment of Pharmacy, University of Parma, Parma, Italy; ^bDepartment of Food Science, University of Parma, Parma, Italy

ABSTRACT

Calcium is recognized as an essential nutritional factor for bone health. An adequate intake is important to achieve or maintain optimal bone mass in particular during growth and old age. The aim of the present study was to evaluate the efficiency of hake fish bone (HBF) as a calcium source for bone mineralization: *in vitro* on osteosarcoma SaOS-2 cells, cultured in Ca-free osteogenic medium (OM) and *in vivo* on young growing rats fed a low-calcium diet. Lithotame (L), a Ca supplement derived from *Lithothamnium calcareum*, was used as control. *In vitro* experiments showed that HBF supplementation provided bone mineralization similar to standard OM, whereas L supplementation showed lower activity. *In vivo* low-Ca HBF-added and L-added diet similarly affected bone deposition. Physico-chemical parameters concerning bone mineralization, such as femur breaking force, tibia density and calcium/phosphorus mineral content, had beneficial effects from both Ca supplementations, in the absence of any evident adverse effect. We conclude HBF derived from by-product from the fish industry is a good calcium supplier with comparable efficacy to L.

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Introduction

Calcium is an essential ion in all organisms and participates in a variety of structural and functional roles. Intracellular calcium, particularly the cytosolic free calcium, is an important second messenger and cofactor for proteins and enzymes regulating key cellular processes such as neurotransmission, motility, hormonal secretion and cellular proliferation. Extracellular calcium, on the other hand, is an integral part of the mineral component of the bone, serves as cofactor for adhesion molecules, clotting factors and other proteins, and modulates neuronal excitability. Regulation of extracellular calcium concentration is under tight endocrine control, which affects the intestinal entry and exit of calcium through the kidneys, using the large skeleton reservoir as buffer, when necessary (Civitelli & Ziambaras 2011). Calcium plays a crucial role in bone health and an adequate calcium intake, together with adequate levels of vitamin D, which are a prerequisite for calcium absorption, has long been recognized to be the most critical nutritional factor for achieving or maintaining optimal bone mass (Anderson et al. 1993; Ross et al. 2011). Although calcium is important for bone health throughout life, calcium needs are particularly

elevated during growth, when bones and muscles develop, and during old age (Mesias et al. 2011; Schulman et al. 2011). Aging is characterized by changes in the endocrine system (e.g. the deficiency of estrogens after menopause in women), in the immune system (aging is associated with a persistent low-grade activation of the innate immune system leading to a proinflammatory status) and by the excessive generation of reactive oxygen species (Schulman et al. 2011). These factors, together with other physiologic changes, such as the reduction in appetite and food intake, the decreased sense of smell and taste, the poor dentition, that predispose to a state of malnutrition termed the “anorexia of aging”, significantly impact bone health, in particular bone remodeling process, leading to an increase in the resorption/formation ratio (Chapman 2007; Schulman et al. 2011). A common condition associated with aging is osteoporosis, characterized by low bone mass and micro-architectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture (Consensus Development Conference 1993). Although the major determinants of peak bone mass and strength are genetic, nutrition plays a crucial role in affecting the ability to achieve peak bone mass and in reducing the risk of osteoporosis: an

adequate calcium, vitamin D and protein intake results in reduced bone remodeling, better calcium retention, reduced age-related bone loss and reduced fracture risk (Peters & Martini 2010; Plawewski & Chapman-Novakofski 2010). In lactose tolerant populations, the greatest amount of dietary calcium is obtained from milk and dairy foods, which also provide the human diet with vitamin D and other important macro- and micronutrients (Caroli et al. 2011). In regions, like Asian countries, where lactose intolerance is widespread among the population, an alternative calcium-rich diet is represented by small fresh water fish eaten whole, including bones. The absorption of calcium from such fish is comparable to that of calcium from skimmed milk both in rats and humans (Hansen et al. 1998; Larsen et al. 2000). Despite the high Ca and P content of fish bones (Toppe et al. 2007) and the huge quantities of this raw material available as by-product from the fish industry, the bone fraction, which comprises approximately 10–15% of the total body weight of fish (skin not included), is still regarded as waste. Recent studies demonstrate that calcium from bigger, commercial fishes like salmon and cod is absorbed as efficiently as CaCO_3 in both growing pigs and humans (Malde et al. 2010a, 2010b). These observations suggest that fish bone is a resource useful, as a high quality food ingredient or supplement, to avoid calcium deficiency and to reduce the risk of osteoporosis.

It is noteworthy that almost 32 million tons of fish tissue are discarded as processing waste of the fishery industry every year, causing a great impact on the environment (Arvanitoyannis & Kassaveti 2008). The reutilization of fish bones in human and animal diet supplementation might have the concomitant positive effect of reducing the undesirable environmental impact of fishing activity (Boutinguiza et al. 2012).

With these premises, the aim of this study was at first to estimate the content and the bioavailability of calcium contained in hake bones flour (HBF) and then to evaluate the efficiency of HBF as a calcium source for bone mineralization both *in vitro* by studying bone formation in immortalized osteosarcoma SaOS-2 cell line, and *in vivo* by monitoring morphological, physico-chemical and biochemical parameters of bone mineralization in growing rats fed a diet containing HBF.

Materials and methods

The fish bones used in these experiments were supplied by “Etale Fishing Co. Ltd” (Walvis Bay, Namibia). Then they were differently processed and grinded to obtain HBF in the laboratories of the Department of Food

Science at the University of Parma, as described in the following section. Lithotame (L), a commercially available calcium supplement, consisting of a multiminerall-rich extract from the red marine algae *Lithothamnium calcareum* (Aslam et al. 2010), was used as an alternative calcium source to compare with HBF. L was chosen as reference compound (Malde et al. 2010b) because the calcified seaweed *Lithothamnium calcareum* is the main constituent of several products for human and animal consumption employed in many countries (Almeida et al. 2012; Cruywagen et al. 2015; O’Driscoll et al. 2013).

Calcium suppliers

Hake bone flour (HBF) preparation

We analyzed two different types of flour obtained from raw or boiled hake bones. Hake bones have been isolated from defrosted hakes using common knives, removing most of the flesh. Since the industrial processing of hake by-products normally gives back boiled bones, half of the bones were boiled in distilled water for 20 min. Both raw and boiled hake bones were dried overnight at 50 °C and subsequently grinded in a mill (HBF).

Hake bone flour (HBF) chemical analysis

The total calcium (Ca), phosphorus (P), sodium (Na), potassium (K) and magnesium (Mg) content of both raw and boiled HBF was determined by Atomic Absorption Spectrometry (Perkin-Elmer, Waltham, MA). The concentration of calcium ions (Ca^{2+}) in the two flour solutions was determined by the colorimetric cresolphthalein complexone kit (cat 64000 FLUKA provided by Sigma Aldrich, Italy). The spectrophotometric measurements were carried out by means of Cary 50 Scan UV-visible spectrophotometer (Varian Inc., Palo Alto, CA). For *in vivo* experiments, boiled HBF was used to supplement low calcium (LC) diets for rat feeding.

Lithotame

Lithotame (Lithotame[®], Phytoitalia) derives from the seaweed “*Lithothamnium calcareum*”. The dried powder of the seaweed is supplied in capsules or tablets *per os* as a dietary supplement of calcium or as antacid. In this study, L was used as a calcium supplement to be compared with HBF. L’s composition is 94.7% ash. Calcium (30%) and magnesium (3%) both as carbonate, are the main minerals contained. L main components are reported in Table 1.

Table 1. Lithotame specifications.

Ash main components	
Calcium	320.01 g/Kg
Magnesium	32.00 g/Kg
Phosphorus	1.06 g/Kg
Sodium	451.5 mg/Kg
Iron	239.8 mg/Kg
Potassium	55.5 mg/Kg

Ash content was determined after calcination in a muffle. Phosphorus was assessed, on samples dissolved in HCl, with colorimetric method. Ca, Mg, Na, K and iron (Fe) were determined, ever on samples dissolved in HCl, by means of an atomic absorption spectrometer (model 1100B; Perkin Elmer, Shelton, CT) (Malacarne et al. 2014, 2015).

For *in vivo* experiments, L was used without any chemical pretreatment to supplement LC diets for rat feeding.

In vitro experiments

Bioavailability

For *in vitro* experiments, raw and boiled HBFs were extracted in HCl acid to mimic acidic gastric environment prior to cell medium supplementation. To this aim, 1 g each of raw and boiled HBF was separately dissolved in 50 ml HCl 1 N under shaking for 30 min at room temperature. The mixtures were then centrifuged at 3500 rpm for 5 min in order to separate the flour solution from the undissolved flour collected in the pellet.

In vitro experiments were carried out by using commercial tablets of L. Since commercial L does not undergo any boiling process prior to oral administration, we avoided this step. However, to mimic the acidic extraction like we did with HBF, L tablets (0.3 g) were at first dissolved in 50 ml HCl 1 N under shaking for 30 min and then centrifuged at 3500 rpm for 5 min, in order to discard the insoluble pellet. The concentration of bioavailable Ca^{2+} in the obtained solution was determined again by means of the colorimetric cresolphaleincomplexone method (see **HBF preparation**).

Cell cultures

Cultures preparation. Human osteosarcoma cells SaOS-2 were provided by Cell Culture Laboratory, Experimental Institute of Zooprophyllaxis of Brescia, Italy. The cells ($10^5/\text{cm}^2$) were routinely passaged and cultured in standard medium (STM): DMEM (PAA Laboratories, Pasching, Austria) supplemented with 10% Fetal Bovine Serum (FBS) (PAA Laboratories, Pasching, Austria), 1% streptomycin/penicillin solution (Sigma-

Aldrich, St. Louis, MO), 1% L-Glutamine (PAA Laboratories, Pasching, Austria), 1% MEM non-essential amino acid solution (Sigma-Aldrich, St. Louis, MO) and 1% RPMI 1640 vitamin solution (Sigma-Aldrich, St. Louis, MO). Cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2 . Medium was changed every 2–3 days. Experimental cultures were created by briefly washing the confluent cell layer with Phosphate Buffered Saline (PBS) (International PbI, Milan, Italy) followed by trypsinization of the cells (Trypsin EDTA, PAA Laboratories, Pasching, Austria). After the formation of a dispersed cell suspension, 200 μl of detached cells were incubated in Trypan blue solution (Sigma-Aldrich, St. Louis, MO) for 5 min. Vital cells were counted in a Bürker's hemocytometer and plated at a density of 2×10^4 cells/ cm^2 into 24 well plates. The cultures were then incubated in STM for 3–4 days. After this period, STM was substituted (day 0) with an osteogenic medium (OM) composed of: STM supplemented with 250 μM L-Ascorbic acid, 10 mM β -glycerophosphate and 10 nM dexamethasone (Sigma-Aldrich, St. Louis, MO). After 2–3 days, we set 4 groups of cells for each culture condition: (1) OM with Calcium-free DMEM (Ca-free OM); (2) Two control mediums (CM) composed of OM with DMEM containing Ca either 0.9 or 1.8 mM; (3) Two Ca-free OM mediums supplemented with Lithotame solution (LM) to obtain 0.9 and 1.8 mM final Ca concentrations; (4) Two Ca-free OM mediums supplemented with HBF solution (HBFM) to obtain 0.9 and 1.8 mM final Ca concentrations. All experiments were triplicated. Mediums were changed every 2–3 days.

Sample collection. The extent of matrix mineralization performed by SaOS-2 in culture was assayed on day 21 by means of the Alizarin Red staining method, according to an adaptation of the protocol described by (Gregory et al. 2004). Cells were washed twice with PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS for 20 min at room temperature and rinsed once with distilled water. After fixation, the cultures were stained with 2% solution of Alizarin Red S (Sigma-Aldrich, St. Louis, MO) in distilled water for 5 min at room temperature under gentle shaking. Excess dye was washed off with distilled water. Considering that Alizarin Red binds selectively to calcium (1 mol dye corresponds to 2 mol calcium) (Schröder et al. 2005) and that the dye is stable at low and high pH (Fain et al. 2004; Niazi et al. 2006), matrix bound Alizarin Red dye has been dissolved in HCl 1 N, transferred in tubes, suitably diluted in NaOH 1 N and its maximum light absorption peak at 270 nm was measured at the UV-Vis spectrophotometer Cary 50 Scan (Varian Inc., Palo Alto, CA).

In vivo experiments

Rat growth

Male 6-week-old Wistar rats (200 ± 5 g) were grown according to Guiding Principles in the Care and Use of Animals (DL116/92). All experimental procedures were approved by the Animal Research Ethics Committee of the University of Parma and were in accordance with the European Union Directive 86/609 and the Italian DLgs 116/92. Young animals that are regularly fed with a standard content of calcium in the diet (0.9%), can grow according to the expected standard conditions reported by the growth chart of “Charles River” supplier. According to the current literature (Iwamoto et al. 2004; Peterson et al. 1992), weight gain can be reduced when these animals are fed with LC diet for two months. In this work, we choose to grow animals with a LC containing diet in order to assess possible differential effect upon the addition of either HBF or L as calcium supplement. Boiled HBF was used to supplement LC diets for rat feeding. L was used without any chemical pretreatment.

To this aim, 15 male rats were randomized in three experimental groups: (1) Low Calcium-group (LCg): five rats were fed low-calcium diet (0.17%); (2) Fish bone-group (HBFg): five rats were fed LC diet with added HBF to restore standard calcium levels (0.9%); (3) Lithotame-group (Lg): five rats were fed LC with added L to restore standard calcium levels (0.9%).

Feed and deionized water were given both ad libitum for eight weeks. The three different diets were prepared by Mucedola SRL (Settimo Milanese, MI, Italy): complete feed for rodent based diet 4RF21 model (LC), or added with HBF, or with L.

Composition of the 4RF21 diet: 66.5% cereals (wheat, wheatstraw, maize and corn gluten feed), 18.2% vegetable protein (soybean meal and yeast), 3.5% animal protein (whey powder), 7.5% forage (lucerne, hazelnut skins), 0.4% fats (soybean oil), 3.2% mono-ammonium phosphate and sodium chloride, 0.1% amino-acids, vitamin and mineral mixture (Table 2).

Analytical constituents and differences between the three diets are reported in Table 3.

Sample collection

Feed intake and body weight were weekly monitored. Moreover, urine and feces produced within 24 h were collected from rats housed in metabolic cages every two weeks to evaluate Ca and P levels (Goulding & McIntosh 1986). Blood (0.5 ml) was collected before the treatment and monthly during the period of observation from sublingual vein of lightly anesthetized rats. Blood

Table 2. Vitaminic and mineral mixture of the three experimental diets.

Additives (per kg)	
E672 Vitamin A	17000 I.U.
E671 Vitamin D3	1290 I.U.
E1 Fe	184 mg
E5 Mn	55 mg
E6 Zn	69 mg
E4 Cu	12 mg
E2 I	0.93 mg
E3 Co	0.65 mg

Table 3. Analytical constituents of the three experimental diets.

	Low Ca-diet	HBF-diet	Lithotame-diet
Analytical constituents %			
Crude protein	19.00	19.50	18.50
Crude oils and fats	2.50	2.50	2.50
Crude fibers	5.50	5.50	5.50
Crude ash	4.50	6.50	6.50

samples were centrifuged (5000 rpm for 10 minutes) to obtain serum. Ca and P contents were spectrophotometrically determined (see **HBF preparation**).

Deoxypyridinoline (DPD), a marker of bone resorption (Mardon et al. 2008) was assessed in basal and final urine samples by using a commercial ELISA kit (Biomedical Assay Co, Ltd.).

After eight weeks feeding period, animals were killed. Femurs and tibias were collected and cleaned from residual connective and muscular tissues. Bones weight was determined using an analytical balance (Mettler AE50) and length was measured in μm using a gauge.

Then femurs were used to evaluate biomechanical properties by means of dynamometer INSTRON[®] according to the method of Zhang and Coon (1997). In particular, each femur was supported by a fulcrum with 1 cm width. A probe with a length of 6 cm and a round base was attached to a 50 kg load cell with a crosshead speed of 5 mm/min in order to evaluate the force (N) required to break femur.

Instead, right tibia was used either to evaluate mineral density (g/cm^3) by means of pycnometric (Sharlab Italia S.r.l.) measurement (Deyhim et al. 2006) and to quantify Ca and P levels by means of atomic absorption spectroscopy (see **HBF preparation**) (Shahnazari et al. 2009). Total bone mineral content was expressed as tibias ash weight/dry weight according to (Peterson et al. 1992). Tibia dry weight was determined after incubation at 180°C for 15 h.

The percent of Ca absorption (a) and retention (b) rates were calculated according to Larsen (2000), respectively, by applying the formulae:

$$(a) \text{ Ca absorption rate (\%)} = (\text{intake Ca} - \text{fecal Ca}) / \text{intake Ca} * 100;$$

Table 4. Calcium, phosphorus, sodium, potassium and magnesium content in raw and boiled hake bone flours (g/kg flour) determined by atomic absorption spectrometry.

Amount (g/kg)	Raw bone flour	Boiled bone flour	<i>p</i>
Calcium	142.33 ± 6.8	180.71 ± 0.63	*
Phosphorus	76.62 ± 0.06	88.25 ± 0.54	*
Sodium	8.86 ± 0.13	5.12 ± 0.34	*
Potassium	13.49 ± 0.25	6.45 ± 0.00	*
Magnesium	2.93 ± 0.01	3.32 ± 0.05	NS

Values are given as mean ± S.D. **p* < 0.05; NS: not significant.

(b) Ca retention (%) = (intake Ca – fecal Ca – urine Ca)/intake Ca * 100.

The percent of P absorption and retention were calculated similarly.

Statistics

Mineral content in HBF: data are analyzed with unpaired, two-tailed *t*-test.

In vitro: data were interpolated with regression lines and standard errors. A specific statistic test (Armitage 1980) was used to compare two by two the slopes of the regression lines.

In vivo: data are presented as means with their standard errors; all data were analyzed with One-way ANOVA followed by the Newman-Keuls post-test or with two-way ANOVA followed by Bonferroni's post-test. Differences were considered significant for *p* < 0.05, very significant for *p* < 0.01 and highly significant for *p* < 0.001.

Results

Determination of mineral content in HBF

Table 4 reports the content of Ca, P, Na, K and Mg in raw and boiled HBF. The quantity of Ca and phosphorous is higher in boiled HBF with respect to raw HBF. On the contrary the content of Na and K is higher in raw bone flour with respect to boiled flour. As regards to Mg, its concentration is the same in the two types of flours. Considering that the quantity of Ca and P is higher in boiled bone flour and that the industrial processing of hake by-products normally gives back boiled bones, we decided to test only boiled HBF in both *in vitro* and *in vivo* experiments.

Experiments in vitro

SaOS-2 cells were seeded in the multi-well plate and cultured for 3–4 days in standard growth medium. During this pre-culture period cells remained in an undifferentiated stage. The substitution of standard growth medium with an osteogenic one inhibited cell

proliferation and induced SaOS-2 differentiation into osteoblast-like cells.

Differentiated SaOS-2 cells were detached, subdivided into groups and grown in different culture conditions for 21 days. During this period, the cells of all groups except those grown in calcium free medium progressively produced an extracellular mineralized matrix, which could be clearly observed by optic microscope. The mineralized matrix was stained with Alizarin Red on day 21. Since this dye binds selectively to Calcium salts, the red spots represent mineralized matrix nodules. Qualitatively speaking, mineralized matrix can be clearly observed in OM, LM and HBFM samples. Instead, only few and small-mineralized matrix nodules are evident in cells grown in Ca-free OM. The staining of OM, LM and HBFM appears much more pronounced when calcium concentration is in the physiological range, i.e. 1.8 mM with respect to 0.9 mM. Moreover, staining appears stronger where cells are grown in OM and HBFM with respect to cells grown in LM. For a quantitative detection of matrix production, matrix bound Alizarin Red was dissolved and spectrophotometrically estimated at 270 nm. The data statistically elaborated in Figure 1 show that the amounts detached from OM and HBFM are significantly equivalent (*p* > 0.05) whereas that of the cells grown in LM is significantly lower than the others (*p* < 0.05).

Experiments in vivo

Rat growth

(a) Rats belonging to the treated experimental groups grew according to the standard growth curve provided by the supplier, whereas LCg shows a significant lower (*p* < 0.01) growth curve starting from 6th week (Figure 2). In particular, a significant increase of body weight in comparison with LCg (*p* < 0.05) was observed for fish bone group at the 8th week of treatment. (b) Groups receiving special diets showed higher daily feed intake (HBFg: 28.80 ± 0.38 g, Lg: 30.00 ± 0.69 g) compared to rats with LC diet (27.22 ± 0.75 g). Daily feed intake was significantly higher for Lg compared to LCg (*p* < 0.05) whereas there was only a slight increase (*p* = 0.05) in total feed intake for Lg in respect to HBF group.

Bone physico-chemical features

(a) Bones weight: Femoral weight was plainly higher for both treated groups with respect to low calcium one (*p* < 0.01), instead no differences were observed for tibia weight among groups (Table 5). (b) Bone mineral content: Ca and P mineral content in tibia was increased

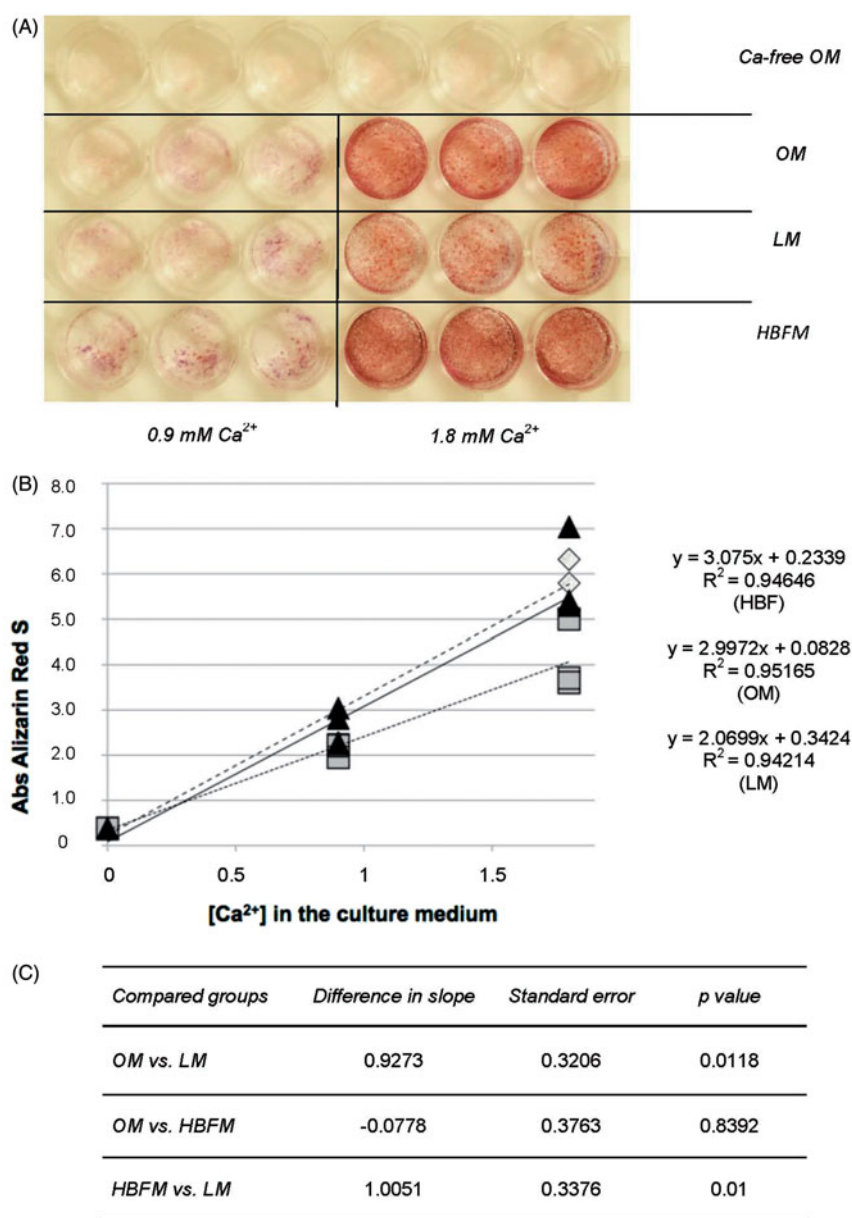


Figure 1. Extracellular mineralized matrix. (A) Mineralized matrix stained with Alizarin Red S. Cells in the first row were grown in calcium free osteogenic medium (Ca-free OM); cells in the second row were grown in standard osteogenic medium (OM); cells in the third row were grown in calcium free osteogenic medium supplemented with Lithotame solution (LM); cells in the fourth row were grown in calcium free osteogenic medium supplemented with hake bone flour solution (HBFM). In the second, third and fourth rows the concentration of calcium in the three wells on the left was 0.9 mM, whereas the concentration of calcium in the three wells on the right was 1.8 mM. (B) Alizarin Red S extracted from the mineralized matrix produced by cells grown in different culture conditions. Squares represent LM cultures; rombs represent OM cultures; triangles represent HBFM cultures. Linear regression line is shown. Its formula is reported on the right of the graph together with the coefficient of determination ($R^2 > 0.94$). (C) The table shows the difference in slope of the regression line of the three culture conditions compared two by two, the standard error and the *p* value obtained from the test used to compare the regression lines. 209 × 297 mm² (222 × 222 DPI).

for both treated groups respect to low calcium one ($p < 0.05$). Ash weight reflects bone mineral content, so the ratio between ash weight and dry weight of tibias were compared among groups. Mineral content was increased ($p < 0.05$) in fish bone group not only with respect to the LCg but also with respect to L (Table 5). (c) Biomechanical properties: Femurs from LC animals

had a statistically lower ($p < 0.05$) breaking force compared to treated groups (Table 5).

Discussion

In order to assess the calcium source quality of fish bone in this study we evaluate the ability of hake bone flour to

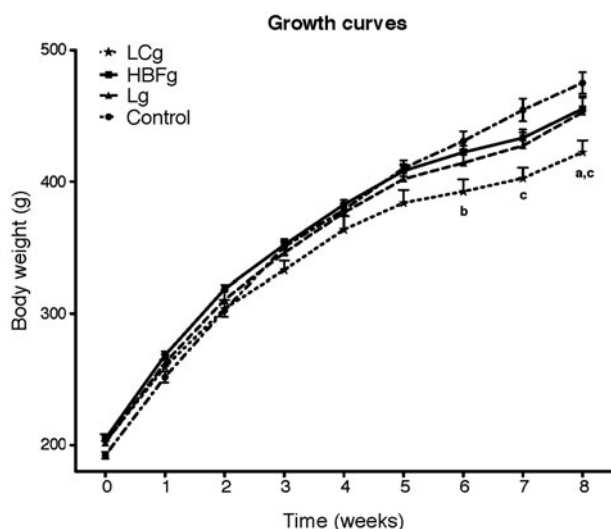


Figure 2. Growth curves. Body weights over time in rats fed diets containing different calcium sources. Data are means \pm SEM, $n=5$. (a) $p<0.05$. Two-way ANOVA Bonferroni post test (LCg vs HBFg). (b) $p<0.01$. (c) $p<0.001$. Two-way ANOVA Bonferroni post test (LCg vs control). LCg: low calcium-group; HBFg: fish bone-group; Lg: lithotame-group; control: reference growth curve for Wistar (Wl) rats (Charles Rivers Laboratories). $209 \times 297 \text{ mm}^2$ ($200 \times 200 \text{ DPI}$).

Table 5. Physical parameters (length, weight and density) and biomechanical properties (breaking force) and Ca and P mineral content of bones of the different experimental groups evaluated at the end of the study.

	LCg	HBFg	Lg
(A) Femur			
Length (cm)	3.489 ± 0.006	3.544 ± 0.025	3.515 ± 0.026
Weight (g)	1.012 ± 0.009	1.128 ± 0.012^b	1.149 ± 0.033^b
Breaking force (N)	105.1 ± 1.9	123.9 ± 4.0^c	130.9 ± 4.2^c
(B) Tibia			
Length (cm)	4.162 ± 0.023	4.137 ± 0.015	4.188 ± 0.038
Weight (g)	0.800 ± 0.025	0.848 ± 0.012	0.865 ± 0.022
Density (g/cm^3)	1.040 ± 0.015	1.077 ± 0.017	1.081 ± 0.023
Mineral Ca (mg)	193 ± 7	205 ± 21^a	213 ± 11^a
Mineral P (mg)	96 ± 4	103 ± 11^a	106 ± 5^a
Mineral content (g/g)	0.592 ± 0.004	$0.608 \pm 0.003^{a,d}$	0.597 ± 0.004

Data are mean \pm SEM, $n=5$.

^a $p<0.05$.

^b $p<0.01$.

^c $p<0.001$ One-way ANOVA, Newman-Keuls post test (treated groups vs LCg).

^d $p<0.05$ One-way ANOVA, Newman-Keuls post test (HBFg vs Lg).

restore matrix mineralization in cell lines and bone mineralization in young growing rats, both in calcium restriction. Results were compared with L, a commonly used calcium supplement.

In vitro experiments evidenced that a better matrix mineralization was exhibited by cells grown in HBF supplemented medium; whereas, a worse effect was observed in L supplemented medium. Noticeably, in fish bones Ca and P are present as apatite-based crystals, with a Ca/P molar ratio of 1.67, similar to that in human

bones. Moreover, in HBF Ca is mainly under the form of phosphate salt with a Ca/P ratio close to physiological requirement, in contrast to L whose calcium salt is mainly a carbonate (CaCO_3). Moreover, the results demonstrated that the composition of HBF is extremely bioavailable and did not exhibit a cytotoxic effect on the cell line. In sum, it may be plausible that bone deposition could be specifically enhanced in wells containing HBF.

As concerns *in vivo* observations, both HBF and L supplemented groups exhibited beneficial effects on bone mineralization in terms of physico-chemical parameters: supplemented diets groups showed increased mineral content of tibia and enhanced weight and fracture load of femur compared to LC group. In accordance with these findings, in literature, dietary calcium supplementation with CaCO_3 prevented the reduction of bone calcium content (Persson et al. 1993; Thomas et al. 1988), bone ultimate load (Thomas et al. 1988) and bone density (Persson et al. 1993) observed in rats consuming low-calcium diet (0.1%). Furthermore in a previous study (Jung et al. 2006) on ovariectomized rats fed LC diet, supplementation with fish bone peptides proved similar beneficial effects on bone mineralization.

In this study both dietary calcium supplements similarly maintained normal growth whereas rats fed low-calcium diet showed a significant decrease of growth curve. It is noteworthy that Lg exhibited a daily feed intake higher than LCg, in accordance with the finding obtained in another study where rats fed a *Lithotamnium calcareum* integrated diet consumed more feed in comparison with the control group (Almeida et al. 2012).

No difference of Ca and P bioavailability of the two suppliers was observed (Table 6); in fact, Ca and P absorption and retention rate were the same. Also, in Malde's study (Malde et al. 2010b) on young healthy men, calcium from two different types of fish bone was absorbed equally well to calcium carbonate.

In our study the changes between the basal and final urinary deoxypyridinoline concentrations showed no statistical differences between animals with calcium-free and calcium-supplemented diets (Table 6). The value of deoxypyridinoline, which is considered a specific marker of bone resorption and osteoclastic activity, remained in the range of the values reported for age-matched normal growing rats (Tanimoto et al. 2003), suggesting that in growing rats changes in calcium diet content does not affect this parameter and indirectly the process of bone resorption.

A further consideration can concern the statistical difference between total mineral content in tibias of HBF supplemented rats compared with those of L supplemented ones. The presence of additional micro and macro-nutrients and metal ions other than Ca and P in

Table 6. Ca and P absorption and retention rates, serum Ca and P levels determined for each experimental group at the end of the study.

Groups	Rate Ca absorption	Rate Ca retention	Rate P absorption	Rate P retention	Serum Ca (mg/dl)	Serum P (mg/dl)	Δ Urinary DPD (nmol/24h)
LCg	91.7 ± 1.8	91.6 ± 1.8	94.8 ± 0.9	93.2 ± 0.8	11.69 ± 0.17	8.61 ± 0.11	0.119 ± 0.036
HBFG	94.9 ± 0.3	94.9 ± 0.3	95.7 ± 0.3	94.6 ± 0.2	11.27 ± 0.05	8.96 ± 0.27	0.136 ± 0.019
Lg	95.7 ± 0.4	95.7 ± 0.4	96.4 ± 0.3	95.5 ± 0.3	11.22 ± 0.04	8.34 ± 0.15	0.132 ± 0.032

Urinary deoxypyridinoline excretion in 24 h is calculated as $\Delta = \text{Final DPD} - \text{Basal DPD}$. Data are mean \pm SEM, $n=5$.

HBFG with respect to L could account for this difference whose relevance in term of bone mineralization and function deserves further investigation.

Lastly, the difference between *in vitro* and *in vivo* results obtained employing the two integrators could merely be derived from the difference in biological complexity and experimental conditions occurring between cells and the entire organisms.

Conclusions

The study on SaOS-2 cells demonstrated that the mineral content of boiled HBFG is bioavailable and did not exhibit a cytotoxic effect on the cell line. The obtained data revealed that fish bone is a suitable calcium supplier with comparable efficacy to L on rat bone mineralization. Furthermore, HBFG could be considered preferable to L for calcium integration because it consents the achievement of growth and bone mineralization comparable to L exploiting what is considered waste material.

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Disclosure statement

The authors declare that they do not have any conflict of interest, either financial or personal relationships with other people or organizations that could influence this work.

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